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(54) Title: PRODUCER CELLS FOR REPLICATION SELECTIVE VIRUSES IN THE TREATMENT OF MALIGNANCY

(57) **Abstract**

The invention includes producer cells for administering to a subject having tumor cells in order to kill tumor cells or prevent growth or spread of the tumor. The producer cell comprises an oncolytic virus which is capable of replicating in the producer cell. The producer cell is not capable of sustained survival in the body of the subject. The invention also includes methods of using these producer cells to treat a subject having tumor cells and making a medicament for use in such treatment.

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PRODUCER CELLS FOR REPLICATION SELECTIVE VIRUSES IN THE TREATMENT OF MALIGNANCY

FIELD OF THE INVENTION

The field of the invention is treatment of malignancy using oncolytic virus agents.

BACKGROUND OF THE INVENTION

Cancer remains one of the leading causes of morbidity and mortality of humans worldwide. Although certain tumors remain localized at discrete locations within the body, at least during certain stages of their growth, other tumors are dispersed from their earliest stages or arise in tissues which line body cavities or organs. For example, epithelial cancers arise in tissues which line the lungs, the ovaries, the exterior of the body, and various body cavities. Epithelial ovarian cancer (EOC) is one such epithelial cancer.

Despite the aggressive surgical approaches and combination chemotherapeutic regimens investigated over the past two decades, EOC remains a disease with a grim prognosis. For example, recent statistics indicate that 25,000 new patients afflicted with EOC are diagnosed yearly in the U.S.; 15,000 deaths occur there from this disease yearly. Unfortunately, due to the lack of symptoms, the majority of patients afflicted with EOC are diagnosed at a late stage. In addition, although 70% of EOC patients initially respond to cisplatin-based chemotherapy, the majority of these patients relapse and develop chemotherapy-resistant disease. As a result, the overall five-year survival rate is approximately 20% for advanced-stage EOC.

Initially it was believed that anti-cancer gene therapy must involve use of replication defective viruses to administer the desired transgene in order to prevent systemic spread of virus and its associated complications, toxicity, or both. Therefore, until recently much of gene therapy for malignant disease has centered on the delivery

of a therapeutic gene (i.e. thymidine kinase, cytosine deaminase, p53, and the like) with replication defective adenoviruses. This method has several potential drawbacks. First, because these vectors are replication defective, it is unclear whether these viruses are able to penetrate more than a few tumor cell layers. Second, introduction of these viruses into humans induces a strong anti-vector/viral immune response, leading to very transient transgene expression.

HIV-1716 is a replication-competent herpes simplex virus type 1 which has a 759-bp deletion in both copies of the *RL1* gene which encodes for the protein ICP34.5, a major determinant of herpes pathogenicity. Viruses with this mutation exhibit drastically reduced neurovirulence. These viruses do not cause encephalitis when inoculated either intracerebrally or peripherally into a host. Moreover, these mutants replicate as well as their wild-type parental strain (e.g. 17+) in a variety of dividing cells lines, but replicate poorly in cells not undergoing mitosis. These characteristics make HSV-1716 and other *RL1* mutants attractive as vectors for cancer gene therapy.

Previous studies have demonstrated that *RL1* mutant herpesviruses like HSV-1716, replicate well in established dividing human glioma cell lines, as well as in primary cell cultures derived from human biopsy material. Infection of these cultures result in cell death in the majority of cases. It is also believed that, in some cell lines, premature shut-off of host protein synthesis occurs in response to a lack of expression of ICP34.5. This has been designated "the double hit phenomenon." *In vivo* studies have also been encouraging. Similar studies have been made with HSV-1 strains which lack ribonuclease reductase activity and strains which are multiply-attenuated (e.g. in which genes encoding ICP34.5 and ribonuclease reductase are deleted). Replication selective adenovirus strains have also been studied. Several groups have shown efficacy in both immunocompromised and immunocompetent mouse models of intracranial malignancies. Further, it has been shown that HSV antigen staining is restricted to the tumor mass with no spread to adjacent normal tissue.

Similar studies have been performed in animal models of malignant mesothelioma, a uniformly fatal neoplasia of the lining of the pleural cavity which does

not respond well to surgery, chemotherapy or radiation. Kucharczuk et al. (1997, Cancer Res. 57:466-471) demonstrated that several non-neuronally derived human cell lines support HSV-1716 growth *in vitro*. Further, their *in vivo* study was based on a well characterized intraperitoneal model of human malignant mesothelioma involving REN cells injected into SCID mice. Their results indicated reduced tumor burden and significantly prolonged survival after intraperitoneal injection of HSV-1716 in tumor-bearing animals. Although malignant mesothelioma lends itself to study because of its location in the lining of the pleural cavity, there is interest in other, more prevalent, thoracic malignancies which have poor prognoses unless identified early. Other malignancies in which morbidity is associated with localized disease include, for example, bronchoalveolar cell, bladder, endometrial, cervical, and ovarian cancers.

Endothelial ovarian cancer (EOC), for example, remains localized within the peritoneal cavity in a large proportion of patients, ultimately causing local morbidity and lethal complications. Because of its localized nature, EOC lends itself to intraperitoneal approaches of therapy. One such approach is gene therapy. Gene therapy comprising either delivering the herpes simplex virus-1 thymidine kinase (HSVtk) suicide gene to diseased cells followed by administration of ganciclovir to the patient or delivering tumor suppressor genes and/or oncogenes to cells has been tested in experimental ovarian cancer models *in vitro* and *in vivo* (Tong et al., 1996, Gynecol. Oncol. 61:175-179; Behbakht et al., Am. J. Obstet. Gynecol. 175:1260-1265; Deshane et al., J. Clin. Invest. 96:2980-2989; Mujoo et al., Oncogene 12:1617-1623). Sufficiently encouraging preclinical results were attained to justify initiation of clinical phase I trials (Link et al., 1996, Human Gene Ther.; Alvarez et al., 1997, Human Gene Ther. 8:597-613). However, results from a recent clinical trial using an adenoviral vector that comprised an HSVtk gene for treatment of mesothelioma localized in the pleural space indicated that adenoviral gene delivery is restricted to a few superficial cell layers and that treatment of larger three-dimensional tumors may be inadequate (Sterman et al., 1998, Human Gene Ther. 9:1083-1092).

Replication-competent and replication-restricted viral agents provide a feasible alternative for cancer therapy. Replication-restricted recombinant attenuated forms of herpes simplex virus-1 (HSV-1) represent one family of such agents (Chambers et al., 1995, Proc. Natl. Acad. Sci. USA 92:1411-1415; Jia et al., 1994, J. Natl. Cancer Inst. 86:1209-1215; Glorioso et al., 1995, Annu. Rev. Microbiol. 49:675-710; Kesari et al., 1995, Lab. Invest. 73:636-648; Kramm et al., 1997, Hum. Gene Ther. 8:2057-2068; Nilaver et al., 1995, Proc. Natl. Acad. Sci. USA 92:9829-9833). For example, HSV-1 mutants have been generated that harbor alterations in genes such as thymidine kinase (tk) or ribonucleotide reductase (RR) and exhibit decreased viral replication in non-dividing neuronal cells. The specificity of the RR^- mutants has been shown to be up to 1,000-fold higher for malignant rodent cells than endogenous neural cells (Boviatsis et al., 1994, Human Gene Ther. 5:183-191).

Another series of HSV-1 mutants has been produced by making alterations in both copies of the *RL1* gene, a diploid fragment of the HSV-1 genome (Chambers et al., 1995, Proc. Natl. Acad. Sci. USA 92:1411-1415; Kramm et al., 1997, Hum. Gene Ther. 8:2057-2068; Mineta et al., 1994, Cancer Res. 54:3963-3966; Pyles et al., 1997, Human Gene Ther. 8:533-544; Randazzo et al., 1995, Virology 211:94-101). Its product, the ICP34.5 protein, has been implicated in neurovirulence and is responsible for preventing apoptosis related to premature shut-off of protein synthesis in the infected host cells. ICP34.5-null HSV-1 mutants have been shown to replicate preferentially in tumor cells, causing a direct oncolytic effect, but appear to spare normal differentiated tissues (Randazzo et al., 1996, Virology 223:392-395; Brown et al., 1994, J. Gen. Virol. 75:3767-3686). These strains have been successfully used to reduce or cure tumors of the central nervous system (CNS) in experimental models (Chambers et al., 1995, Proc. Natl. Acad. Sci. USA ; Jia et al., 1994, J. Natl. Cancer Inst. 86:1209-1215; Kesari et al., 1995, Lab. Invest. 73:636-648; Kramm et al., 1997, Hum. Gene Ther. 8:2057-2068; Nilaver et al., 1995, Proc. Natl. Acad. Sci. USA 92:9829-9833; Martuza et al., 1991, Science 252:854-856; Mineta et al., 1994, Cancer Res. 54:3963-3966; Mineta et al., 1995, Nature Med. 1:938-943; Boviatsis et al., 1994,

Human Gene Ther. 5:183-191; Pyles et al., 1997, Human Gene Ther. 8:533-544; Randazzo et al., 1995, Virology 211:94-101; Andreansky et al., 1997, Cancer Res. 57:1502-1509; Yazaki et al., 1995, Cancer Res. 55:4752-4756). Thus, the use of these vectors alone to kill tumor cells is accepted in the art.

The efficacy of HSV-1716, an ICP34.5 null mutant of HSV-1, for reducing tumor burden and conferring survival advantage has been demonstrated in an intraperitoneal model of malignant mesothelioma in severe combined immunodeficient (SCID) mice (Kucharczuk et al., 1997, Cancer Res. 57:466-471). Moreover, these studies suggested that extra-CNS administration of replication-restricted HSV-1 is safe. HSV-1716 administered intraperitoneally to SCID mice appeared to be completely avirulent. In fact, there was no viral spread outside the tumors, as was documented in Kucharczuk et al. (1997, Cancer Res. 57:466-471) by immunohistochemistry and polymerase chain reaction (PCR) analysis of multiple murine tissues, including intraperitoneal and retroperitoneal organs as well as distant organs and the brain. This was not true for wild-type HSV-1, to which SCID mice were found to be extremely sensitive. In fact, intraperitoneal administration of wild-type HSV-1 to SCID mice led to rapid systemic spread of the virus and death of the animals within one week. Furthermore, administration of HSV-1716 to normal human skin in a murine xenograft model was accompanied by no toxicity, while administration of a wild-type HSV-1 led to rapid destruction of the xenograft (Randazzo et al., 1996, Virology 223:392-395).

Use of replication-restricted HSV-1 for extra-CNS malignancies was recently extended to other tumors. A ribonuclease reductase-deleted mutant was used in an experimental animal model of metastatic colorectal carcinoma of the liver (Carroll et al., 1996, Ann. Surg. 224:323-329). In separate experiments, a replication-restricted ICP34.5 mutant was used to treat experimental metastatic and subcutaneous melanoma (Randazzo et al., 1995, Virology 211:94-101; Randazzo et al., 1997, J. Invest. Dermatol. 108:933-937). In addition, a multi-attenuated mutant, HSV-G207, was efficacious for treatment of breast cancer (Toda et al., 1998, Human Gene Ther. 9:2173-2185).

Taken together, these studies demonstrate that use of various oncolytic viruses to kill tumor cells is well accepted, even if prior art uses of such oncolytic vectors have been plagued with shortcomings such as low efficacy, low tissue specificity, rapid clearing of oncolytic viruses, and inability to deliver a sufficiently high or prolonged doses of virus to the desired tumor tissue. The present invention includes producer cells and methods of using them that overcome the shortcomings of the prior art, thereby permitting efficacious delivery of oncolytic viruses to tumor tissue and effective treatment of cancers such as EOC.

BRIEF SUMMARY OF THE INVENTION

The invention relates to a producer cell for administration to a subject having tumor cells. The producer cell comprises an oncolytic virus which is capable of replicating in the producer cell. The producer cell, however, is incapable of sustained survival in the body of the subject. In one embodiment, the oncolytic virus is cytotoxic with respect to the producer cell in the body of the subject. In another embodiment, the producer cell is rendered incapable of sustained survival in the body of the subject by exposing the producer cell to a lethal dose of radiation. The lethal dose of radiation may be a dose which enhances the burst size of the producer cell (e.g. about 3 Gray). In yet another embodiment, the producer cell is rendered incapable of sustained survival in the body of the subject by incorporating a suicide gene (e.g. thymidine kinase or cytosine deaminase) into the producer cell.

In one aspect of the invention, the producer cell exhibits binding affinity for a tumor cell in the subject, such as an epithelial tumor cell (e.g. an epithelial ovarian cancer cell).

In another aspect of the invention, the oncolytic virus is capable of replicating in a tumor cell of the subject. For example, the oncolytic virus may be less capable of replicating in a non-tumor cell of the subject than in the tumor cell.

In yet another aspect, the oncolytic virus may incapable of replicating in a non-tumor cell of the subject, or it may be incapable of replicating in any cell of the subject. Replication of the oncolytic virus may, for example, be under the control of a

tumor-associated transcriptional promoter such as the prostate specific antigen promoter or the tumor growth factor-β promoter.

The producer cell may, for example, be selected from the group consisting of a PA-1 cell, an REN cell, a PER.C6 cell a 293 cell, a melanoma cell, a glioma cell, and a teratocarcinoma cell. Preferably, the producer cell is a PA-1 cell.

The oncolytic virus may, for example, be selected from the group consisting of a herpes simplex virus-1, a herpes simplex virus-2, an adenovirus, a vesicular stomatitis virus, a Newcastle disease virus, and a vaccinia virus. When the oncolytic virus is a herpes simplex virus-1, it preferably does not express functional ICP34.5. Suitable herpes simplex virus-1 include, but are not limited to, HSV-1716, HSV-3410, HSV-3616, HSV-R3616, HSV-R47, HSV-G207, HSV-7020, HSV-NVR10, HSV-G92A, HSV-3616-IL-4, and HSV-hrR3. Suitable herpes simplex virus-2 include, but are not limited to, strain 2701, strain 2616, and strain 2604. Suitable adenoviruses include, but are not limited to, ONYX-15, Ad5d1520, Ad5d1312, CN706, Addl110, Addl111, Addl118, and Addl004.

In another aspect, the producer cell further comprises a composition selected from the group consisting of an immunomodulatory molecule, a cytokine, a targeting molecule, a cell growth receptor, an immunoglobulin which is specific for the tumor, a nucleic acid encoding an immunomodulatory molecule, a nucleic acid encoding a cytokine, a nucleic acid encoding a targeting molecule, a nucleic acid encoding a cell growth receptor, and a nucleic acid encoding an immunoglobulin which is specific for the tumor.

The invention also relates to an anti-tumor agent comprising a mammalian cell which comprises thymidine kinase. The mammalian cell exhibits binding affinity for a tumor cell in a human patient and is incapable of sustained survival in the body of the patient. When the mammalian cell is administered to the patient, the mammalian cell binds with a tumor cell in the patient. When gancyclovir is thereafter administered to the patient, the mammalian cell metabolizes gancyclovir to generate a cytotoxic metabolite which is provided to the tumor cell with which the mammalian cell has bound.

The invention further relates to a method of killing tumor cells in a mammal. This method comprises administering to the mammal a producer cell. The producer cell comprising a oncolytic virus which is capable of replicating in the producer cell. The producer cell is incapable of sustained survival in the body of the mammal. The mammal may, for example, be a human afflicted with an epithelial cancer or a human afflicted with an tumor.

The invention still further relates to use of a producer cell for manufacture of a medicament for administration to a patient having tumor cells. The producer cell comprises a oncolytic virus which is capable of replicating in the producer cell. However, the producer cell is incapable of sustained survival in the body of the patient.

DETAILED DESCRIPTION

The invention relates to a producer cell for administration to a subject (e.g. a human patient) having tumor cells. The producer cell comprises any of a wide variety of oncolytic viruses (e.g. the herpes simplex virus-1 mutant designated HSV-1716). The oncolytic virus is capable of replicating in the producer cell, and may also be capable of replicating in tumor cells in the subject. The producer cell is not capable of sustained survival in the body of the subject. Because the producer cell supports replication of the oncolytic virus, it may contain many (e.g. tens, hundreds, or thousands) of copies of the virus. When the producer cell is administered to a subject, the copies of the virus escape from the cell and are delivered (e.g. by fluid-mediated dispersion or by producer cell-to-tumor cell contact) to tumor cells in the subject. Once delivered to tumor cells in the subject, the oncolytic viruses kill the tumor cells.

Delivery of oncolytic viruses using producer cells has advantages over prior art direct injection methods of delivering such viruses to tumor cells. For example, because the virus is, in some embodiments, capable of replicating in the producer cell of the invention, the amount of virus which can be administered in a given volume of fluid can be greatly increased, since cells in which a virus has replicated may contain tens, hundreds, or even thousands of copies of the virus. In

addition, delivery of a virus within a producer cell may enable the virus to elude the subject's immune system, increasing the likelihood that the virus will reach and kill a tumor cell. Furthermore, if the producer cell used to deliver the oncolytic virus exhibits binding affinity for tumor cells in a subject, delivery of the virus using the producer cell will increase localization of the virus to the tumor cells in the patient. This may be particularly important for treatment of tumors that are not discretely localized. Other advantages of the producer cells and the methods of using them, as described herein, will be apparent to the skilled artisan in view of the present disclosure.

Definitions

As used herein, each of the following terms has the meaning associated with it in this section.

The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

A "subject" is an animal, preferably a mammal such as a human.

A subject "has tumor cells" if the subject comprises or is suspected to comprise tumor cells in any form (i.e. in the form of a solid tumor, a dispersed tumor, a metastatic tumor cell, or the like).

A tumor cell is "killed" if it is induced to lyse, if it is induced to undergo apoptosis, or if it is rendered incapable of growing or dividing.

An "oncolytic virus" is any virus which is able to kill a tumor cell by infecting the tumor cell.

A virus is "cytotoxic with respect to" a cell if the virus is able to kill the cell after infecting the cell.

An "anti-tumor agent" is a composition of matter which, when applied to a tumor cell, kills the tumor cell.

A "transcriptional promoter" is a nucleic acid which, when operably linked with a second nucleic acid encoding a gene product such as an RNA or a protein, enables the gene product to be expressed in a cell by virtue of permitting an RNA polymerase enzyme to transcribe the second nucleic acid.

By describing two polynucleotides as "operably linked" as used herein is meant that a single-stranded or double-stranded nucleic acid moiety comprises each of the two polynucleotides and that the two polynucleotides are arranged within the nucleic acid moiety in such a manner that at least one of the two nucleic acid sequences is able to exert a physiological effect by which it is characterized upon the other.

A "suicide gene" is a gene which, when expressed in a cell, induces lysis or apoptosis of the cell or renders the cell incapable of growth or division.

Replication of a virus is "under the control of a promoter" if at least one gene product required for replication for replication of the virus is operably linked with the promoter.

A cell "exhibits binding affinity" for a tumor cell if the cell binds to the tumor cell with greater affinity than the affinity with which it binds to a non-tumor cell.

As used herein, a "functional" biological molecule is a biological molecule in a form in which it exhibits a property by which it is characterized. A functional enzyme, for example, is one which exhibits the characteristic catalytic activity by which the enzyme is characterized.

An oncolytic virus is "replication-selective" if it is more capable of replicating in an tumor cell of a subject than in a non-tumor cell of the subject.

Description

A goal of viral gene therapy for treatment of malignant disease has been delivery of a therapeutic/suicide gene (e.g. thymidine kinase, cytosine deaminase, p53, etc.) to tumor cells in a subject. In many prior art methods, such genes are delivered to tumor cells using a replication defective virus, such as an adenovirus. Use of replication competent or replication selective viruses for the treatment of neoplastic disease provides certain advantages. Such viruses deliver the desired gene to a larger percentage of tumor cells. Furthermore, replication of these viruses *in vivo* may well be oncolytic in their own right.

One group of viruses which are useful for gene therapy are replication competent herpes simplex type 1 viruses (HSV-1) having deletions in the *RL1* gene, which encodes the protein designated ICP34.5. This protein is a major determinant of

herpes pathogenicity. Viruses having mutations in this gene exhibit reduced neurovirulence 105 . For example, they do not cause encephalitis when inoculated either intracerebrally or peripherally. Moreover, these mutants replicate as well as their wild-type parental strain (e.g. 17+) in a variety of dividing cells lines, but replicate poorly in cells not undergoing mitosis. These characteristics make these HSV-1 mutant viruses attractive for use as oncolytic viruses.

In an effort to increase the efficacy of such oncolytic vectors, the present inventors have devised a virus delivery method whereby cells that are infected with the mutant herpesvirus serve as "producer cells" for an oncolytic virus. Use of producer cells to deliver oncolytic viruses *in vivo* provides several advantages. For example, rapidly dividing cell lines have the ability to replicate viruses very efficiently, producing as many as about 6,000 copies of a virus per infected cell. The producer cells are, in effect, viral factories which can increase the effective dose of virus administered to the patient. Administering producer cells may protect, at least initially, the virus from neutralizing host immunity. Producer cell lines may be engineered to enhance tumor killing, for example, by selecting or designing producer cells which produce cytokines which enhance the oncolytic effects of the virus with which they are infected. Oncolytic gene therapy has, until now, focused on localized malignancies where tumors can be directly injected with vector (e.g. glioma) or where vector can be instilled into a discrete body cavity (e.g. the pleural cavity for malignant mesothelioma). The producer cell of the invention may be used to treat both localized and diffuse or disseminated malignancies. In addition, the presence of producer cells may have a positive effect on tumor killing by inducing an immune response against the tumor cells.

The invention includes a producer cell for administration to a subject having tumor cells. In one aspect, the producer cell comprises a suicide gene (e.g. thymidine kinase) and exhibits binding affinity for a tumor cell. When administered to a patient, the producer cell binds with a tumor cell in the subject, if one is present. Expression of the suicide gene, optionally coupled with administration to the subject of a substrate of an enzyme encoded by the suicide gene (e.g. ganciclovir when the suicide

gene is thymidine kinase) leads to death of the producer cell and, by virtue of a bystander effect, to cells located near the producer cell. Thus, if the producer cell has bound with a tumor cell in the subject, death of the producer cell induces death of the bound tumor cell.

In an important aspect of the invention , the producer cell comprises an oncolytic virus. The oncolytic virus is capable of replicating in the producer cell, but the producer cell is incapable of sustained survival in the body of the patient. Thus, according to this embodiment the producer cell makes many copies of the oncolytic virus, and releases them into the subject's body upon the death of the producer cell. In another embodiment, the oncolytic virus is not capable of replicating in the producer cell, but is nonetheless carried into and released within the body of the subject by the producer cell.

The producer cell of the invention is preferably one of many types of cells which are known to exhibit binding affinity for tumor cells, but it is not necessary that the producer cell exhibit such affinity. For example, the producer cell may be a PA-1 cell, an REN cell, a PER C6 cell, a 293 cell, a melanoma cell, a glioma cell, or a teratocarcinoma cell. Also preferably, the producer cell is obtained from an animal of the same species (and strain, if applicable) as the subject or from a cell line derived from such an animal. Preferably, the producer cell is well tolerated by the subject (i.e. is not rejected by the immune system of the subject) when the producer cell is injected into the patient. By way of example, acceptable producer cells for use in human patients include any human cell line which is derived from a human source and which does not induce hyperacute rejection when injected into the patient. Alternatively acceptable producer cells for use in humans include any cell line which has been engineered to not induce hyperacute rejection.

The producer cell is not capable of sustained survival in the subject's body, by which is meant that the producer cell does not endure more than several months, several weeks, or several days in the body of the subject following administration of the producer cell to the subject. Preferably, the producer cell is not capable of replicating in the subject's body. Numerous methods are known in the art

for rendering cells incapable of sustained survival in a subject, and any of those methods may be used to so render the producer cell of the invention. For example, the oncolytic virus of the invention may be selected such that it is cytotoxic with respect to the producer cell in the body of the subject. Thus, the virus kills not only tumor cells in the patient, but also the producer cells which are used to deliver the virus. The producer cell may also be rendered incapable of sustained survival in the body of the patient by exposing the producer cell to a lethal dose of radiation prior to providing the producer cell to the subject. For example, a radiation dose of 20 Gray will kill nearly all known cells which might be used as producer cells. Furthermore, it is understood that certain lethal doses of radiation enhance the burst size of the oncolytic virus in the producer cell. For example, it has been found that when the dose of radiation is about 3 Gray, the burst size of the HSV-1 variant G207 in PA-1 cells is enhanced, relative to non-irradiated cells of the same type. Furthermore, the producer cell may be rendered incapable of sustained survival in the body of the patient by incorporating a suicide gene (e.g. thymidine kinase or cytosine deaminase) into the producer cell. Upon expression of the suicide gene (optionally coupled with administration to the subject of a substrate of an enzyme encoded by the suicide gene, such as ganciclovir when the suicide gene is thymidine kinase), the producer cell is killed. Potential undesirable immune reactions may be minimized or avoided by using producer cells which are incapable of sustained survival in the subject.

The producer cell of the invention preferably exhibits binding affinity for a tumor cell in the patient. Use of such producer cells has a number of benefits. For example, binding of a producer cell to a tumor cell necessarily brings the oncolytic virus(es) in or on the producer cell into close association with the tumor cell, increasing the likelihood that the virus will infect and kill the tumor cell. Furthermore, it is known that at least certain viruses may be transmitted by cell-to-cell contact. Thus, binding of a producer cell and a tumor cell may enhance targeting of virus to the tumor cell in this manner as well. In addition, formation of a producer cell-tumor cell complex may generate or expose antigenic regions which can be recognized by the subject's immune system, leading to generation of an immune response against the tumor cells. If the

producer cells are engineered to contain, display, or express various immune modulators, growth factors, or suicide genes, binding of the producer cell to a tumor cell in the subject localized the biological activity of these molecules to the tumor site. For example, the producer cell may comprising an immunomodulatory molecule, a cytokine, a targeting molecule, a cell growth receptor, an immunoglobulin which is specific for the tumor, or a nucleic acid encoding one of these. The cell may either naturally comprise one of these molecules or be engineered to comprise the molecule.

Preferably, the producer cell exhibits binding affinity for an epithelial tumor cell, such as an epithelial ovarian cancer cell. Because epithelial tumors (like dispersed tumors such as various leukemias) are not necessarily present at a single focal site in the body of a subject, prior art gene therapy methods have experienced difficulty delivering the gene vector to all tumor sites. If the producer cell binds with one of these dispersed or widely spread tumor types, then the oncolytic virus of the invention will be delivered to the tumor cells wherever they are dispersed or spread.

The oncolytic virus of the invention may be substantially any virus which is known to exhibit oncolytic activity and which is capable of replicating in, or at least being carried by, a producer cell of the invention without ablating the oncolytic activity of the virus. In one embodiment, the oncolytic virus of the invention is also able to replicate in a tumor cell of the patient, and is preferably less capable of replicating in a non-tumor cell of the patient than in a tumor cell of the patient. For example, the oncolytic virus may incapable of replicating in a non-tumor cell of the patient. Of course, so long as the virus is oncolytic, it may be incapable of replicating in any cell of the subject.

An oncolytic virus may be made replication-selective if replication of the virus is placed under the control of a regulator of gene expression such as, for example, a minimal enhancer/promoter region derived from the 5'-flank of the human *PSA* gene (e.g. see Rodriguez et al., 1997, Cancer Res. 57:2559-2563). By way of example, the main transcriptional unit of an oncolytic virus may be placed under transcriptional control of the tumor growth factor-β (TGF-β) promoter by operably linking virus genes to the TGF-β promoter. It is known that certain tumor cells

overexpress TGF-β, relative to non-tumor cells of the same type. Thus, an oncolytic virus wherein replication is subject to transcriptional control of the TGF-β promoter is replication-selective, in that it is more capable of replicating in the certain tumor cells than in non-tumor cells of the same type. Similar replication-selective oncolytic viruses may be made using any regulator of gene expression which is known to selectively cause overexpression in an affected cell. The replication-selective oncolytic virus may, for example, be an HSV strain in which a gene encoding ICP34.5 is mutated. The oncolytic virus of the invention may also be one which exhibits binding affinity for a tumor cell of the subject.

The oncolytic virus of the invention may, for example, be a herpes simplex virus-1, a herpes simplex virus-2, an adenovirus, a vesicular stomatitis virus, a Newcastle disease virus, or a vaccinia virus. Examples of such oncolytic viruses are described, for example, in Kim (1999, In: Gene Therapy of Cancer, Academic Press, San Diego, CA, pp. 235-248).

When the oncolytic virus of the invention is a herpes simplex virus-1, it is preferably one which does not express functional ICP34.5 protein (e.g. HSV-1716) or one of the HSV-1 viruses described in Coukos et al., (1998, Gene Ther. Mol. Biol. 3:79-89). Exemplary HSV-1 viruses include HSV-1716, HSV-3410, HSV-3616, and HSV-4009. Other replication selective HSV-1 virus strains which may be used as the oncolytic virus of the invention include, by way of example and not limitation, HSV-R3616 (in which the gene encoding ICP34.5 is deleted), HSV-R47 (in which genes encoding proteins R3616 and ICP47 are deleted), HSV-G207 (in which genes encoding ICP34.5 and ribonucleotide reductase are deleted), HSV-7020, HSV-NVR10 (in which genes encoding 7020 and ICP47 are deleted), HSV-3616-UB (in which genes encoding ICP34.5 and uracil DNA glycosylase are deleted), HSV-G92A (in which the albumin promoter is a transcriptional regulated promoter), HSV-3616-IL-4, HSV-hrR3 (in which the gene encoding ribonucleotide reductase is deleted) and HSV strains which do not express functional ICP34.5 and which express a cytokine such as interleukin-2, interleukin-4, or GM-CSF.

When the virus is a herpes simplex virus-2, it may, for example, be selected from the group consisting of strain 2701, strain 2616, and strain 2604.

When the virus is an adenovirus, it may, for example, be selected from the group consisting of ONYX-15, Ad5d1520, Ad5d1312, CN706, Addl110, Addl111, Addl118, and Addl004.

The producer cells of the invention may be used in a method of killing tumor cells in a mammal. The method comprising administering to the mammal a producer cell comprising a oncolytic virus which is capable of replicating in the producer cell. The mammal may be substantially any mammal having tumor cells, and is preferably a human patient afflicted with a localized cancer such as malignant mesothelioma, EOC, bladder cancer, or the like.

The producer cells of the invention may be selectively engineered to enhance their organ- or cell-specificity as well as their ability to induce tumor killing. Known gene products may be used enhance the oncolytic effect of viruses delivered to tumor cells by the producer cells of the invention. For example, increased levels of certain cytokines (e.g. interleukins 2, 4, and 6, γ-interferon, and tumor necrosis factor α) inhibit tumor growth and metastasis. Producer cells which secrete such a cytokine in conjunction with virus production will have a synergistic effect on tumor killing. Producer cell lines may similarly be modified to enhance immune recruitment to the area of administration or decrease the effect of pre-existing immunity to the vector.

Preliminary experiments have demonstrated an inhibitory effect of ascites fluid on viral cell killing *in vitro*. This is thought to be due to the presence of IgG in the ascites. It is known that particular viral glycoproteins (gE & gI) have the ability to bind the Fc portion of antibodies; thus preventing the antibodies from binding antigens on the surface of cells and activating complement. It is also known that glycoprotein gC has the ability to inactivate complement directly. A producer cell which produces such glycoproteins can absorb and inactivate antibody in ascitic or pleural fluid, decreasing inhibition of viral spread.

The invention also includes use as producer cells of genetically-modified cells which express a suicide gene such as HSV-*TK* and exhibit binding

affinity for tumor cells. These cells are sensitive to ganciclovir because they convert ganciclovir into various toxic metabolites. These cells exhibit therapeutic effect on tumor cells, based on the "bystander effect," which is death of cells adjacent to the genetically-modified cells. The bystander effect is thought to be caused by transport of one or more toxic metabolites of ganciclovir from cell to cell through gap junctions as well as by induction of an immune response.

Pharmaceutical Compositions

The invention encompasses the preparation and use of medicaments and pharmaceutical compositions comprising the producer cell of the invention as an active ingredient. Such a pharmaceutical composition may consist of the active ingredient alone, in a form suitable for administration to a subject, or the pharmaceutical composition may comprise the active ingredient and one or more pharmaceutically acceptable carriers, one or more additional ingredients, or some combination of these. Administration of one of these pharmaceutical compositions to a subject is useful for killing tumor cells or arresting tumor growth or spread in the subject, as described elsewhere in the present disclosure.

As used herein, the term "pharmaceutically acceptable carrier" means a chemical composition with which the active ingredient may be combined and which, following the combination, can be used to administer the active ingredient to a subject.

The formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with a carrier or one or more other accessory ingredients, and then, if necessary or desirable, shaping or packaging the product into a desired single- or multi-dose unit.

Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for ethical administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to animals of all sorts.

Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and perform such modification with merely ordinary, if any, experimentation. Subjects to which administration of the pharmaceutical compositions of the invention is contemplated include, but are not limited to, humans, primates, and other mammals.

Pharmaceutical compositions that are useful in the methods of the invention may be prepared, packaged, or sold in formulations suitable for intraperitoneal, oral, rectal, vaginal, parenteral, topical, pulmonary, intranasal, buccal, ophthalmic, or another route of administration. Other contemplated formulations include projected nanoparticles, liposomal preparations, and immunologically-based formulations.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

Controlled- or sustained-release formulations of a pharmaceutical composition of the invention may be made using conventional technology.

Liquid suspensions, for example, may be prepared using conventional methods to achieve suspension of the active ingredient in an aqueous or oily vehicle. Aqueous vehicles include, for example, water and isotonic saline. Oily vehicles include, for example, almond oil, oily esters, ethyl alcohol, vegetable oils such as arachis, olive, sesame, or coconut oil, fractionated vegetable oils, and mineral oils such as liquid paraffin. Liquid suspensions may further comprise one or more additional ingredients including, but not limited to, suspending agents, dispersing or wetting agents, emulsifying agents, demulcents, preservatives, buffers, salts, flavorings,

coloring agents, and sweetening agents. Oily suspensions may further comprise a thickening agent. Known suspending agents include, but are not limited to, sorbitol syrup, hydrogenated edible fats, sodium alginate, polyvinylpyrrolidone, gum tragacanth, gum acacia, and cellulose derivatives such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose. Known dispersing or wetting agents include, but are not limited to, naturally-occurring phosphatides such as lecithin, condensation products of an alkylene oxide with a fatty acid, with a long chain aliphatic alcohol, with a partial ester derived from a fatty acid and a hexitol, or with a partial ester derived from a fatty acid and a hexitol anhydride (e.g. polyoxyethylene stearate, heptadecaethyleneoxycetanol, polyoxyethylene sorbitol monooleate, and polyoxyethylene sorbitan monooleate, respectively). Known emulsifying agents include, but are not limited to, lecithin and acacia.

A pharmaceutical composition of the invention may also be prepared, packaged, or sold in the form of oil-in-water emulsion or a water-in-oil emulsion. The oily phase may be a vegetable oil such as olive or arachis oil, a mineral oil such as liquid paraffin, or a combination of these. Such compositions may further comprise one or more emulsifying agents such as naturally occurring gums such as gum acacia or gum tragacanth, naturally-occurring phosphatides such as soybean or lecithin phosphatide, esters or partial esters derived from combinations of fatty acids and hexitol anhydrides such as sorbitan monooleate, and condensation products of such partial esters with ethylene oxide such as polyoxyethylene sorbitan monooleate. These emulsions may also contain additional ingredients including, for example, sweetening or flavoring agents.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for rectal administration. Such a composition may be in the form of, for example, a suppository, a retention enema preparation, and a solution for rectal or colonic irrigation.

Suppository formulations may be made by combining the active ingredient with a non-irritating pharmaceutically acceptable excipient which is solid at

ordinary room temperature (i.e. about 20°C) and which is liquid at the rectal temperature of the subject (i.e. about 37°C in a healthy human). Suitable pharmaceutically acceptable excipients include, but are not limited to, cocoa butter, polyethylene glycols, and various glycerides. Suppository formulations may further comprise various additional ingredients including, but not limited to, antioxidants and preservatives.

Retention enema preparations or solutions for rectal or colonic irrigation may be made by combining the active ingredient with a pharmaceutically acceptable liquid carrier. As is well known in the art, enema preparations may be administered using, and may be packaged within, a delivery device adapted to the rectal anatomy of the subject. Enema preparations may further comprise various additional ingredients including, but not limited to, antioxidants and preservatives.

A pharmaceutical composition of the invention may be prepared, packaged, or sold in a formulation suitable for vaginal administration. Such a composition may be in the form of, for example, a suppository, an impregnated or coated vaginally-insertable material such as a tampon, a douche preparation, or a solution for vaginal irrigation.

Methods for impregnating or coating a material with a chemical composition are known in the art, and include, but are not limited to methods of depositing or binding a chemical composition onto a surface, methods of incorporating a chemical composition into the structure of a material during the synthesis of the material (i.e. such as with a physiologically degradable material), and methods of absorbing an aqueous or oily solution or suspension into an absorbent material, with or without subsequent drying.

Douche preparations or solutions for vaginal irrigation may be made by combining the active ingredient with a pharmaceutically acceptable liquid carrier. As is well known in the art, douche preparations may be administered using, and may be packaged within, a delivery device adapted to the vaginal anatomy of the subject.

Douche preparations may further comprise various additional ingredients including, but not limited to, antioxidants, antibiotics, antifungal agents, and preservatives.

As used herein, "parenteral administration" of a pharmaceutical composition includes any route of administration characterized by physical breaching of a tissue of a subject and administration of the pharmaceutical composition through the breach in the tissue. Parenteral administration thus includes, but is not limited to, administration of a pharmaceutical composition by injection of the composition, by application of the composition through a surgical incision, by application of the composition through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration is contemplated to include, but is not limited to, subcutaneous, intraperitoneal, intravenous, intraarterial, intramuscular, or intrasternal injection and intravenous, intraarterial, or kidney dialytic infusion techniques.

Formulations of a pharmaceutical composition suitable for parenteral administration comprise the active ingredient combined with a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. Such formulations may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold in unit dosage form, such as in ampules, in multi-dose containers containing a preservative, or in single-use devices for auto-injection or injection by a medical practitioner. Formulations for parenteral administration include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and implantable sustained-release or biodegradable formulations. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In one embodiment of a formulation for parenteral administration, the active ingredient is provided in dry (i.e. powder or granular) form for reconstitution with a suitable vehicle (e.g. sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition.

The pharmaceutical compositions may be prepared, packaged, or sold in the form of an injectable aqueous or oily suspension. This suspension or solution may

be formulated according to the known art, and may comprise, in addition to the active ingredient, additional ingredients such as the dispersing agents, wetting agents, or suspending agents described herein. Such injectable formulations may be prepared using a non-toxic parenterally-acceptable diluent or solvent, such as water or 1,3-butane diol, for example. Other acceptable diluents and solvents include, but are not limited to, Ringer's solution, isotonic sodium chloride solution, and fixed oils such as synthetic mono- or di-glycerides. Other parentally-administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form, in a liposomal preparation, or as a component of a biodegradable polymer systems. Compositions for sustained release or implantation may comprise pharmaceutically acceptable polymeric or hydrophobic materials such as an emulsion, an ion exchange resin, a sparingly soluble polymer, or a sparingly soluble salt.

As used herein, "additional ingredients" include, but are not limited to, one or more of the following: excipients; surface active agents; dispersing agents; inert diluents; granulating and disintegrating agents; binding agents; lubricating agents; sweetening agents; flavoring agents; coloring agents; preservatives; physiologically degradable compositions such as gelatin; aqueous vehicles and solvents; oily vehicles and solvents; suspending agents; dispersing or wetting agents; emulsifying agents, demulcents; buffers; salts; thickening agents; fillers; emulsifying agents; antioxidants; antibiotics; antifungal agents; stabilizing agents; and pharmaceutically acceptable polymeric or hydrophobic materials. Other "additional ingredients" which may be included in the pharmaceutical compositions of the invention are known in the art and described, for example in Genaro, ed., 1985, Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, which is incorporated herein by reference.

Another aspect of the invention relates to a kit comprising a pharmaceutical composition of the invention and an instructional material. As used herein, an "instructional material" includes a publication, a recording, a diagram, or any other medium of expression which is used to communicate the usefulness of the pharmaceutical composition of the invention for killing tumor cells in a subject, for

preparing producer cells of the invention using one or more components of the kit, or for administering the producer cells of the invention to a subject. The instructional material of the kit of the invention may, for example, be affixed to a container which contains a pharmaceutical composition of the invention or be shipped together with a container which contains the pharmaceutical composition. Alternatively, the instructional material may be shipped separately from the container with the intention that the instructional material and the pharmaceutical composition be used cooperatively by the recipient.

The invention also includes a kit comprising a pharmaceutical composition of the invention and a delivery device for delivering the composition to a subject. By way of example, the delivery device may be a squeezable spray bottle, a metered-dose spray bottle, an aerosol spray device, an atomizer, a dry powder delivery device, a self-propelling solvent/powder-dispensing device, a syringe, a needle, a tampon, or a dosage measuring container. The kit may further comprise an instructional material as described herein.

The invention is now described with reference to the following Examples. These Examples are provided for the purpose of illustration only and the invention should in no way be construed as being limited to these Examples, but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Example 1

In order to develop an effective method of vector delivery, EJ-6-2-Bam-6a cells, an NIH 3T3 fibroblast cell line, were used as producer cells for HSV-1716 in a immunocompetent murine model of lung cancer. It was demonstrated that Lewis Lung Carcinoma (LLC), a spontaneous non-immunogenic murine lung cancer, is sensitive to treatment using HSV-1716 *in vitro* as well as *in vivo*.

LLC supported replication of and was efficiently lysed by HSV-1716 at a multiplicity of infection (MOI) of 1.0 (<20% of cells were viable four days following infection). HSV-1716 replicated in LLC cells with a burst size of 20. In

EJ-6-2-Bam-6a, the virus replicated with significantly greater efficiency, exhibiting a burst size of about 2,200.

It was hypothesized that producer cells would facilitate viral delivery in an *in vivo* system. To test this hypothesis, cell mixing studies were performed using LLC cells injected into the flanks of C57BL/6 mice. These syngeneic cells, a fraction of which were infected with HSV-1716, completely prevented tumor formation by non-infected LLC cells in 10 animals when the cells were mixed at an infected:non-infected ratio of 1:10 (P=0.002). Significant growth inhibition was also seen at a ratio of 1:100. The mean tumor weight in mice administered non-infected LLC cells was 0.092 gram, and tumors in the 1:100 treated group averaged 0.026 gram (P=0.001).

Analogous experiments using EJ-6-2 cells in place of LLC cells in this model, demonstrated similar results at an infected:non-infected ratio of 1:10 (P=0.007) and effective growth retardation at a ratio as low as 1:1000 (tumor weight of 0.224 gram in controls versus 0.072 gram in mice administered a 1:1000 ratio of EJ-6-2 cells P=0.02). Without wishing to be bound by any particular theory of operation, it is believed that the tumor growth-inhibiting effects was attributable to a dual effect of the virus-infected EJ-6-2 cells. First, EJ-6-2 cells have a much greater burst size than the LLC, thus drastically increasing the effective dose of virus administered (MOI). Second, studies involving irradiated EJ-6-2 cells which were not infected with HSV-1716 also demonstrated significant tumor suppression (tumor weight of 0.224 gram in control mice versus 0.051 gram in mice administered irradiated EJ-6-2 1:10; p=0.03), suggesting that EJ-6-2 cells elicited an immune response to this normally non-immunogenic tumor.

Based on these data, we conclude that HSV-1716 is useful as a new gene therapy vector for, at least, lung cancer and that the use of allogeneic producer cells such as EJ-6-2 can enhance the oncolytic efficacy of virus both by increasing the effective MOI of the virus and by inducing an inflammatory response within the tumor milieu.

Example 2

Use of Producer Cells to Deliver a

Replication-Selective Herpes Simplex Virus-1 Mutant

for Intraperitoneal Therapy of Epithelial Ovarian Cancer

Epithelial ovarian cancer (EOC) remains localized within the peritoneal cavity in many patients, lending itself to intraperitoneal therapeutic approaches. In the experiments presented in this Example, the effect of intraperitoneally administering a replication-selective herpes simplex virus-1 (rsHSV-1) on EOC cells was assessed. The rsHSV-1 was administered by direct injection of virus particles and by injection of producer cells which had been infected using the rsHSV-1. Irradiated human teratocarcinoma PA-1 cells were used as the producer cells.

The rsHSV-1 used in the experiments in this Example, HSV-1716, is a replication-competent attenuated strain of HSV-1 which does not express ICP34.5. Contacting EOC cells with HSV-1716 *in vitro* induced dose-dependent oncolysis. A single intraperitoneal administration of 5×10^6 plaque-forming units (pfu) of HSV-1716 resulted in significant reduction of tumor volume and tumor spread and increase in survival in a mouse xenograft model of EOC. PA-1 cells support HSV-1716 replication *in vitro* and bind preferentially to human ovarian carcinoma surfaces, relative to mesothelial surfaces, both *in vitro* and *in vivo*.

In comparison to intraperitoneal administration of HSV-1716 alone, intraperitoneal administration of irradiated PA-1 cells infected with HSV-1716 induced significantly greater tumor reduction in the two xenograft models tested. Significant prolongation of mean survival associated with injection of HSV-1716-infected cells, but not associated with injection of HSV-1716 alone, was also observed in one model. Histologic evaluation indicated the presence of extensive necrosis at tumor sites infected with HSV-1716. Immunohistochemistry to detect HSV-1716 indicated that areas of viral infection were present within tumor nodules, and that such infection persisted for several weeks following treatment. Administration of HSV-1716-infected producer cells induced more widespread infection of tumor tissue by the virus.

The experiments presented in this Example indicate that replication-competent attenuated HSV-1 exerts a potent oncolytic effect on EOC, and that this oncolytic effect may be enhanced by administering the virus in the form of virus-infected producer cells. Without wishing to be bound by any particular theory of operation, it is believed that the enhanced oncolytic effect of producer cell-delivered virus, relative to virus alone, is attributable to amplification of the number of virus particles delivered to tumor tissue and to preferential binding of producer cells to tumor surfaces.

The materials and method used in the experiments presented in this Example are now described.

Virus

Isolation of HSV-1716 has been described previously (MacLean et al., 1991, J. Gen. Virol. 72:631-639). The genome of this virus contains a 759-base-pair deletion located within each copy of the *Bam*H1 fragment of the long repeat region of the genome. These deletions encompass most of the gene encoding ICP34.5. The mutant therefore does not express this protein. Passage of the virus has also been described (MacLean et al., 1991, J. Gen. Virol. 72:631-639; Kucharczuk et al., 1997, Cancer Res. 57:466-471).

Cells

Epithelial ovarian cancer cell lines SKOV3, NIH:OVCAR3, CaOV3, and human ovarian teratocarcinoma line PA-1 have been previously described, and were obtained from the American Tissue Culture Collection (Plainview, MD; Tainsky et al., 1988, Anticancer Res. 8:899-913). The A2780 EOC cell line was obtained from the Fox Chase Cancer Center (Philadelphia, PA).

Primary ovarian cultures were obtained from patients afflicted with advanced EOC, at stages III or IV, according to the criteria set by the International Federation of Gynecologists and Obstetricians (DiSaia et al., 1993, In: Clinical Gynecologic Oncology, Mosby-Year Book, Inc., St. Louis, MO). Malignant effusions, obtained at the time of exploratory laparotomy or diagnostic/therapeutic paracentesis,

were centrifuged at 300×g for 10 minutes at room temperature, and the cell pellets were collected and seeded in standard tissue culture media, as described herein. These cells assumed an epithelial phenotype and their malignant nature was confirmed by a rapid doubling time (average 18 hours) and their immortalized behavior *in vitro*. EOC cells were passaged 4-5 times prior to using them in experiments.

Normal peritoneal mesothelial cells were obtained from intraoperative pelvic peritoneal lavages carried out using normal saline in patients undergoing laparotomy for benign pelvic pathology (e.g. pelvic relaxation, uterine myomata). Lavage fluids were centrifuged at 300×g for 10 minutes at room temperature and the cell pellets were collected and seeded in standard media (see below). These cells assumed an epithelial-like phenotype and grew in a cobblestone pattern. Their doubling time was longer than that of primary EOC cultures (average 36 hours), and their non-malignant nature was confirmed by the fact that they propagated for only a few (4-5) passages even in the presence of growth factors.

To eliminate macrophages from primary cultures, culture media were aspirated 30 min following plating, and suspended cells were re-seeded in new culture flasks. All cell lines and EOC primary isolates were cultured under standard conditions (37°C in a 5% CO_2 atmosphere) in RPMI media comprising 10% (v/v) heat-inactivated fetal calf serum (FCS) and antibiotics. For normal mesothelial cells, media were supplemented with a mixture of growth factors (SerXtend™, Irvine Scientific, Santa Anna, CA) at 0.1% dilution.

Assessment of Cytotoxicity *in vitro*

Cells were incubated in 96-well plates at a density of 3×10^3 cells per well. The cells were incubated in the presence of HSV-1716 at multiplicities of infection (MOI) of 0.1 and 1 in serum-free media for one hour. Serum-enriched media was subsequently added and cultures were observed for four days. Cell proliferation assays were performed using a chromogenic kit (CellTiter AQueous96™, Promega, Madison, WI) and colorimetric assays were performed using a microplate ELISA

reader (Bio-Tek Instruments, Winooski, VT). Cytopathic effects (CPE) were documented by phase microscopy.

Flow Cytometry Analysis of HSV Antigens

PA-1 cells were cultured in T25 flasks until they reached 60% confluence. The cells were then infected with 0.5-2.5 MOI of HSV-1716, as described above. Cells were harvested at 16 hours using a 0.05% trypsin solution, washed once with phosphate buffered saline (PBS) and fixed and permeabilized using 70% methanol at -20°C for 20 minutes. Cells were labeled using a polyclonal antibody (obtained from American Qualex, La Mirada, CA) which specifically binds with HSV-1 proteins, which was used at a dilution of 1:250, and a secondary anti-rabbit fluorescein isothiocyanate (FITC)-conjugated antibody (obtained from Jackson Immunoresearch Laboratories Inc., West Groove, PA), which was used at a dilution of 1:250. Flow cytometric analysis was performed using an EPICS™ XL flow cytometer (Coulter Corporation, Hialeah, FL).

One-Step Growth Curves

PA-1 teratocarcinoma cells were incubated overnight in 6-well plates at a density of 4×10^5 cells per well under standard culture conditions and were then infected with HSV-1716 at 0.3 MOI. In parallel experiments, PA-1 cells were subjected to a single 20 Gray dose of ionizing radiation one hour prior to infection with HSV-1716. Cells were harvested in the accompanying medium by mechanical scraping one hour after infection with the virus (designated '0 hours') as well as 6, 20, 24, and 48 hours later and stored at -80°C. One-step growth curves were generated as described (Kucharczuk et al., 1997, Cancer Res. 57:466-471).

In vivo Adhesion Assays

PA-1 teratocarcinoma cells were labeled using a rhodamine fluorescent dye (PKH26 Red Fluorescent Cell Linker Kit, Sigma Chemical Co., St. Louis, MO) as recommended by the manufacturer. Briefly, cells were harvested using a 0.05% (w/v) trypsin solution, re-suspended in PBS, and incubated with a 1:250 dilution of the dye at for 8 minutes. The labeling reaction was termination by addition of 100% FCS. Cells

were washed, suspended in RPMI media containing 10% FCS, and injected intraperitoneally (at 5×10^6 cells per animal) into SCID mice having intraperitoneal SKOV3 tumors, as described below.

Eight hours after PA-1 cell injection, the animals were sacrificed, and the parietal peritoneum was entirely dissected into four specimens. Each of the specimens contained almost entirely one of the four different segments of the parietal peritoneum (i.e. the diaphragmatic segment, the ventral segment, the lateral left segment, or the lateral right segment). In addition, random biopsies were obtained from the mesentery and the visceral peritoneum. Six micrometer sections were prepared, mounted in Fluoromount™ medium (Fisher, Pittsburgh, PA) containing 2.5% 1,4-diazabicyclo-(2,2,2)octane (DABCO, Polyscience, Warrington, PA) to prevent fluorescent quenching, and examined using a Zeiss microscope.

For quantitative analysis of *in vivo* adhesion, 20 random fields per slide (at 40× magnification) were inspected on each of five slides cut 18 micrometers apart from each other from each of the four different segments of the parietal peritoneum and from each of two sections of mesenteric areas. Fields were selected in such a way that they contained only tumor surface or tumor-free normal peritoneum. The number of fields containing SKOV3 tumor with or without adhering fluorescent PA-1 cells and those containing normal peritoneum with or without adhering fluorescent PA-1 cells were counted.

In vitro Adhesion Assays

Normal peritoneal mesothelial cells, PA-1 teratocarcinoma cells, and EOC cells of type SKOV3, CaOV3, NIH:OVCAR3, or A2780 were incubated in 48-well plates at a density of 3×10^4 to 4×10^4 cells per well and allowed to form 100% confluent monolayers. Teratocarcinoma PA-1 cells cultured in T25 flasks were starved with methionine-free media for 2 hours and then incubated overnight with ^{35}S-methionine-containing media (50 microcuries per milliliter) containing 1% dialyzed FCS. Radio-labeled PA-1 cells were harvested by short exposure to 0.05% trypsin solution, washing once with serum-free media, centrifugation at 300×g for 5

minutes at room temperature, and re-suspension in media containing 1% FCS. PA-1 cells were then seeded at 5×10^3 per well on cell monolayers and allowed to interact with adherent mesothelial or ovarian cancer cells for 30 minutes.

Following co-incubation of PA-1 and mesothelial or cancer cells, the wells were washed three times with PBS to remove non-adherent cells. Adherent cells were harvested using 0.1% trypsin solution, transferred to scintillation vials, and enumerated using a scintillation counter (model LS 6500, Beckman Instruments, Fullerton, CA).

In separate experiments, PA-1 teratocarcinoma cells were radio-labeled using ^{35}S-methionine, as described above, and subjected to ionizing radiation (20 Gray) and/or infection with HSV-1716 (at 2 MOI). Cells subjected to these treatments were permitted to interact with the different monolayer substrates, and adhesion was measured, as described above.

In vivo Xenograft Model of Epithelial Ovarian Cancer

Six- to eight-week-old female CB17 SCID mice (Charles River, Wilmington, MA) were housed in an isolation unit. A2780 and SKOV3 cells were produced using standard culture conditions until they reached 70% confluence. The cells were harvested using 0.05% (w/v) trypsin solution, washed with serum-enriched media, and centrifuged at 300×g for 5 minutes at 4°C. About 5×10^6 SKOV3 cells or 1×10^6 A2780 cells per animal were injected intraperitoneally in 0.5 milliliter of RPMI medium containing 10% FCS and 1% SerXtend™ (Irvine Scientific). Five mice were sacrificed at selected times to confirm the presence of intraperitoneal tumors prior to administering treatment in each experiment. At the end of the experiments, animals were sacrificed and intraperitoneal tumors were assessed for spread and volume.

A semi-quantitative scoring system was devised to assess tumor spread. Five areas were screened for the presence of tumor:

1) the injection site;

2) the parietal peritoneum and diaphragm;

3) the small bowel mesentery and omentum;

4) the lesser omentum and hepatic hilum; and

5) the retroperitoneum.

Each site was assigned a score of 0-3 as following: "0" if no microscopic tumor was seen; "1" if one or more microscopic tumors were visible with the aid of a dissecting microscope at 2.5× magnification; "2" if tumor nodules less than 5 millimeters in diameter were visible; and "3" if tumor nodules equal to or more than 5 millimeters in diameter were present. The presence of ascites resulted in addition of one point in the scoring system. The maximum score accumulated in this scale was therefore 16. All visible tumor nodules were dissected from surrounding normal peritoneum and viscera, and the total weight of intra-abdominal tumor was determined for each animal.

Utilization of PA-1 Teratocarcinoma Cells as HSV-1716 Producer Cells

PA-1 human teratocarcinoma cells were exposed to ionizing radiation at a single dose of 20 Gray and were then infected with HSV-1716 at an MOI of 2. Two hours after infection, cells were washed with PBS and harvested using a 0.05% (w/v) trypsin solution. Cells were washed twice in media comprising 10% heat-inactivated FCS, centrifuged at 300×g for 5 minutes, and re-suspended in RPMI media comprising 1% heat-inactivated FCS. About 5×10^6 cells were injected intraperitoneally into tumor-bearing animals, as described below. Control tumor-bearing animals received PA-1 teratocarcinoma cells that had been exposed to the same amount of radiation but that were not infected with HSV-1716. To confirm that irradiated PA-1 cells did not cause tumorigenicity, non tumor-bearing mice received a flank (n=10) or an intraperitoneal (n=10) administration of 5×10^6 irradiated (but not infected) PA-1 cells and were observed for 16 weeks.

Application of HSV-1716 *in vivo*

At selected times, animals received a single intraperitoneal dose of 5×10^6 plaque-forming units (pfu) HSV-1716 at in 500 milliliters of serum-free RPMI medium. This dose was previously determined to be effective to reduce mesothelioma tumor load (Kucharczuk et al., 1997, Cancer Res. 57:466-471). Control animals received a similar volume of virus-free medium. A separate group of animals received

an intraperitoneal injection of 5×10^6 pfu of irradiated PA-1 teratocarcinoma cells which had been infected with HSV-1716 at an MOI of 2, as described above. A second control group received irradiated, but non-infected, PA-1 cells.

Animals were sacrificed at selected times and intraperitoneal tumors were assessed in the animals. For survival experiments, animals were injected with tumor and treated as above. Animals were observed daily and removed from the cages, if found dead, or sacrificed, if they appeared severely ill. Tumor weight was estimated in sacrificed animals as described above. Survival experiments were repeated twice for each EOC cell line. Kaplan-Meier survival curves were computed utilizing the StatView™ computer program.

Histology and Immunohistochemistry

Tumors obtained from treated and control animals were immediately fixed in formalin (3.7% (v/v) formaldehyde in PBS) and embedded in paraffin. Six-micrometer-thick sections were de-paraffinized and stained using hematoxylin-eosine (H&E). For immunohistochemical analysis, the slides were subjected to antigen retrieval at 105°C for 10 minutes in 0.1 normal citric acid and incubated with a monoclonal antibody which binds specifically with HSV-1 (DAKO, Carpenteria, California) at a dilution of 1:4 for 30 minutes at 47°C. A horseradish peroxidase-conjugated anti-mouse antibody (Vectastain, Vector Laboratories, Burlingame, CA) was used at a dilution of 1:400. Slides were processed using the ABC™ Kit (Vector Laboratories) according to the manufacturer's instructions. Mouse pre-immune serum was used at a dilution of 1:4 as a negative control (DAKO, Carpenteria, California).

Statistical Analysis

Differences in values obtained during *in vitro* adhesion assays and differences in tumor weights in animal experiments were determined using one-way ANOVA. Post-hoc comparisons of specific paired groups were performed using the t-test. Differences in *in vivo* adhesion assays were computed using the Chi-square test for a 2×2 contingency table. Odds ratios (OR) were computed using the formula

OR=a×d/b×c from the 2×2 contingency tables. Survival curves were analyzed using the Mantel-Cox Logrank test. Statistical significance was set at $p < 0.05$. Results are expressed as the mean ± standard error.

The results of the experiments presented in this Example are now described.

HSV-1716 Exerts an Oncolytic Effect on Epithelial Ovarian Cancer *in vitro*

In order to assess the oncolytic effect of HSV-1716 on EOC cells *in vitro*, primary EOC cultures and established EOC cell lines were exposed to HSV-1716 at 0.1 and 1 MOI. Cell survival was assessed by proliferation colorimetric assays and evaluation of cytopathic effect (CPE), as assessed by phase-contrast microscopy. HSV-1716 exerted a direct dose-dependent cytolytic effect on all EOC cell lines and on human teratocarcinoma PA-1 cell line.

Primary EOC cultures obtained from patients afflicted with advanced disease displayed 10- to 20-fold higher sensitivity to cytolysis by HSV-1716 than established EOC cell lines. EOC cell line A2780 was the most sensitive of the cell lines tested, and SKOV3 was the least sensitive of those tested. Teratocarcinoma PA-1 cells were highly sensitive to HSV-1716-induced cytolysis. The sensitivity of PA-1 cells was comparable to that of primary EOC cultures.

These sensitivity observations were confirmed by phase microscopy. 100% of primary EOC cells, A2780 cells, and teratocarcinoma PA-1 cells exhibited CPE within 24-48 hours of exposure at HSV-1716 at 1 MOI. About 95-99% of NIH:OVCAR3, SKOV3, and CaOV3 cells exhibited CPE within four days of exposure.

HSV-1716 Exerts an Oncolytic Effect on Epithelial Ovarian Cancer *in vivo*

In order to assess the efficacy of HSV-1716 for treating established intraperitoneal EOC, a murine xenograft model of EOC was used together with two well characterized EOC cell lines, namely cell lines SKOV3 and A2780. These two cell lines were selected because, in addition to being well characterized, these two cell lines exhibited the least and greatest, respectively, sensitivity to cytolysis by HSV-1716

in vitro. The oncolytic effect of HSV-1716 was evaluated by examining xenografted mice having different tumor burdens, by varying the duration of treatment, and by using the two aforementioned cell lines to generate the tumors, thereby yielding intraperitoneal tumors having different volumes.

To assess the effect of HSV-1716 in minimally-spread EOC, the virus was administered one week following the injection of SKOV3 ovarian cancer cells into mice. Intraperitoneal injection of 5×10^6 SKOV3 cells resulted in formation of microscopic tumor(s) at the diaphragm and occasionally at the omentum, mesentery, or lesser omentum within one week. The tumor score (as described above) for these mice was 2/16. The tumor weight in these mice (n=5) was 202 ± 7.1 milligrams.

One week after injecting SKOV3 cells into the mice, a group (n=10) of animals received a single dose of HSV-1716, while a group of control animals (n=10) received medium only. Animals were 5 weeks later to evaluate tumors. Animals that received virus displayed significantly smaller tumors compared to control animals ($p<0.001$), as indicated in Table 1. Moreover, HSV-treated animals displayed tumors similar to their pre-treatment counterparts ($p=0.78$). Tumor spread was advanced in untreated animals (tumor score 15.5/16), while it remained similar to pre-treatment tumor spread in the HSV-treated animals (tumor score 2.2/16).

Table 1

		Tumor Weight, milligrams		
	Week during which Treatment Began	Pre-Treatment	Non-Treated (at week 6)	HSV-1716 (at week 6)
SKOV3 Tumors	1	202 ± 7	1,147 ± 11	191 ± 1[A]
	3	837 ±9	1,249 ± 13.2	732 ± 3[A]
A2780 Tumors	1	1,800 ±511	18,705 ± 2,120	2,870 ± 923[A]
	3	8,900 ± 921	19,100 ± 1,115	10,032 ± 1,812[B]

Notes:

[A] $p<0.001$

[B] $p<0.05$

In order to assess the role of HSV-1716 in more advanced EOC, xenografted SKOV3 tumors were allowed to grow for three weeks before the mice were injected with virus. At three weeks and without virus treatment, larger intraperitoneal tumors were observed (tumor score: 3.5/16; tumor weight: 837 ± 9.1 milligrams; n=5) than the tumors observed following only one week of growth. Virus-treated animals received a single dose of HSV-1716 intraperitoneally, and control animals received only medium. Animals were sacrificed at 6 weeks (i.e. three weeks after treatment) to evaluate tumors. Animals that received HSV-1716 (n=10) displayed significantly smaller tumors compared to control animals (n=10; $p<0.001$). Moreover, HSV-treated animals displayed similar tumors to their pre-treatment counterparts ($p=0.86$). Tumor spread was again advanced in untreated animals (tumor score 16/16), while it remained similar to pre-treatment in the HSV-treated animals (tumor score 3.7/16).

In order to assess the role of HSV-1716 in bulky EOC, the A2780 cell line was injected into mice. Administration of 1×10^6 A2780 cells resulted in growth of bulky intraperitoneal tumors characterized by the presence of tumor nodules having diameters of from 0.5 to 2 millimeters in diameter throughout the abdominal cavity within one week (tumor score 16/16; tumor weight: 1860 ± 532 milligrams; n=5). One

week after injection of A2780 cells, one group of animals (n=10) received a single dose of HSV-1716, and a group of control animals (n=10) received medium only. Animals were sacrificed 5 weeks later in order to evaluate tumors. HSV-treated animals displayed significantly smaller tumors than control animals that received saline at week one ($p<0.001$; n=10). No difference in tumor weight was detected between the HSV-treated animals and their pre-treatment counterparts ($p=0.86$; n=10).

In order to assess the role of HSV-1716 in more widespread bulky intraperitoneal EOC, A2780 tumors were allowed to grow for three weeks following cell injection. At that time, tumor nodules having diameters of from 4 to about 14 millimeters were observed throughout the abdominal cavity of each mouse (tumor score 16/16; tumor weight: 8150 ± 912 mg; n=5). Administration of a single dose of HSV-1716 three weeks following cell injection resulted in arrest of tumor growth at 6 weeks (three weeks later), relative to the pre-treatment counterpart mice. HSV-treated animals displayed significantly smaller tumors at 6 weeks, compared to control animals that received media ($p<0.05$; n=10). In addition, no significant differences in tumor weight were observed between the animals treated with HSV-1716 at week three and their pre-treatment counterparts ($p=0.66$; n=10).

HSV-1716 Efficiently Infects and Replicates in Irradiated PA-1 Cells

A difficulty of comparing the effect of virus administered alone and virus administered by way of producer cells relates to controlling the number of particles inoculated. To minimize the difference in the virus load initially administered to xenografted mice, the lowest MOI at which 100% of PA-1 cells were infected with HSV-1716 was determined. PA-1 cells were incubated with increasing doses of the virus and the rate of infection of the cells was determined by flow cytometry. These experiments indicated that approximately 100% of PA-1 cells were infected at an MOI of 2. At this MOI, each PA-1 cell presumably was initially infected by one or at most two viral particles.

It was hypothesized that producer cells would lead to significant amplification of the viral load delivered intraperitoneally to xenografted mice *in vivo*.

To assess the magnitude of viral replication in PA-1 cells *in vitro*, the viral burst size (i.e. the number of infectious virus particles released from a PA-1 cell upon cytolysis following infection of the cell with the virus and incubation) was determined. The burst size of HSV-1716 in PA-1 teratocarcinoma cells was 200 in the absence of ionizing radiation. Because administration of HSV-1 infected producer cells into humans will most likely be performed after eliminate the risk of administering potentially uninfected producer cells (i.e. by irradiating the cells), PA-1 cells were subjected to a single (lethal) dose of 20 Gray one hour prior to infection, and viral replication in the irradiated cells was assessed. Burst size experiments indicated that irradiated PA-1 cells infected with HSV-1716 supported viral replication with a burst size of 70 following irradiation at 20 Gray. It was furthermore found that irradiation of the cells infected with HSV-G207 using a radiation dose of 3 Gray significantly increased the burst size, relative to non-irradiated cells.

PA-1 Producer Cells Preferentially Bind to Epithelial Ovarian Cancer Surface Compared to Normal Peritoneum *in vivo*

In order to assess the behavior of the producer cells in the murine xenograft model, fluorescently labeled PA-1 cells were injected intraperitoneally to SKOV3 tumor-bearing mice. Overall, a large number of fluorescent PA-1 cells adhered to areas of the diaphragm or other peritoneal surfaces that were covered by tumor. In some areas, PA-1 cells almost formed a monolayer covering the SKOV3 preexisting tumors. In the absence of tumor nodules, there were few and isolated fluorescent PA-1 cells attaching to normal peritoneal surfaces.

When the number of tumor fields containing adherent PA-1 cells were compared to the number of non-tumor peritoneum fields containing adherent PA-1 cells, it was observed that there was a significantly higher frequency of binding of PA-1 cells to tumor surfaces than to normal peritoneum. Specifically, 90 random diaphragmatic areas harboring SKOV3 tumor cells were examined and 91.1% displayed adherent PA-1 cells, while among 110 diaphragmatic areas with no tumor cells, only 18.1% displayed PA-1 adherent cells ($p<0.0001$, OR=49.9), as indicated in

Table 2A. If only the areas displaying more than 3 PA-1 cells were considered, 91.1% of tumor-positive and 6.3% of tumor-negative microscopic fields had PA-1 cells bound thereto ($p<0.0001$, OR=158.4). Similar results were obtained analyzing lateral or ventral parietal peritoneum and mesenteric peritoneum. Among 89 areas examined that harbored SKOV3 tumor, 75.3% had adherent PA-1 cells, while among 241 areas with no tumor inspected, only 14.1% had adherent PA-1 cells ($p<0.0001$, OR=18.6). In these tissues, if only the microscopic fields displaying more than 3 PA-1 cells were considered, 79.8% of tumor-positive and 9.9% of tumor-negative microscopic fields displayed PA-1 cells ($p<0.0001$, OR=36.1). These results are tabulated in Table 2B.

Table 2A

	Tumor Cells Present in the Field	Tumor Cells Not Present in the Field
PA-1 Cells Bound to the Field	82 (91.1%)	20 (18.2%)
PA-1 Cells Not Bound to the Field	8 (8.9%)	90 (81.8%)

Table 2B

	Tumor Cells Present in the Field	Tumor Cells Not Present in the Field
PA-1 Cells Bound to the Field	67 (75.3%)	34 (14.1%)
PA-1 Cells Not Bound to the Field	22 (24.7%)	207 (85.9%)

PA-1 Producer Cells Preferentially Bind to Epithelial Ovarian Cancer Surface Compared to Normal Peritoneum *in vitro*

The observed differences between PA-1 cell binding to tumor and non-tumor cells *in vivo* could have been related to molecular factors controlling cell-cell interactions between PA-1 cells and murine mesothelial cells. Alternatively, these differences have been related to physical forces governing peritoneal fluid circulation, which might direct both SKOV3 tumor cells and PA-1 cells towards the same peritoneal sites. In addition, these differences have been caused by decreased affinity

of PA-1 teratocarcinoma cells to murine tissues as compared to human tissues. In order to analyze the interaction of PA-1 cells with human EOC and normal peritoneal cells, *in vitro* adhesion assays were performed to compare adhesion of PA-1 teratocarcinoma cells to normal human peritoneum mesothelial cells, several EOC cell lines, and primary EOC cultures. These experiments indicated that PA-1 teratocarcinoma cells displayed significantly higher binding to ovarian cancer cells than to normal human mesothelium ($p<0.001$ for each mesothelial culture versus each ovarian cancer primary culture or cell line except A2780). To assess the effect of radiation or HSV infection on PA-1 adhesion, adhesion of PA-1 teratocarcinoma cells to different ovarian cancer cell lines following radiation was compared with adhesion following infection with HSV-1716 and adhesion after both radiation treatment and infection with virus. These treatments did not significantly affect PA-1 adhesion to cancer cells or normal mesothelial cells.

PA-1 Producer Cells Effectively Deliver HSV-1716 *in vivo*

In order to assess the suitability of producer cells for delivering HSV-1716 particles to tumor cells *in vivo*, direct administration of HSV-1716 (i.e. via injection of a suspension of virus particles) was compared with administration of PA-1 cells infected with HSV-1716. One week following the administration of A2780 tumor cells, SCID mice received a single intraperitoneal administration of either virus alone or HSV-infected PA-1 cells. Control animals received medium or non-infected, irradiated PA-1 cells, respectively. Four weeks later, a significant increase in tumor weight was noted in control animals (20 ± 2.5 grams; $n=20$) and in animals which received non-infected, irradiated PA-1 cells (22.3 ± 3 grams; $n=20$), relative to pre-treatment animals (1.9 ± 0.1 grams; $n=5$; $p<0.001$ for both). Administration of HSV-1716 alone ($n=20$) allowed tumors to grow slightly, as evidenced by a small increase in tumor weight (3.4 ± 0.2 grams), relative to pre-treatment animals ($p<0.05$). Injection of PA-1 cells infected with HSV-1716 ($n=20$) inhibited tumor growth (tumor weight = 2 ± 0.2 grams), relative to pre-treatment animals ($p=0.198$). Tumors in HSV-treated animals and in those treated with HSV-infected producer cells were

significantly smaller than those of their control ($p<0.01$ and $p<0.001$, respectively). Tumors in animals treated with HSV-infected producer cells were significantly smaller than those in animals which were treated with HSV-1716 alone ($p<0.05$).

Similar results were obtained in experiments performed using SKOV3 cells injected intraperitoneally (5×10^6 cells per animal) into SCID mice, as indicated in Table 3. One week following injection of tumor cells, control animals received medium (group 1, n=20) or irradiated, non-infected PA-1 cells (group 2, n=20). Virus-treated animals received a single intraperitoneal injection of virus (group 3, n=20) or HSV-infected PA-1 producer cells (group 4, n=20), respectively (Table 3). Animals from each group were sacrificed at 4 and 7 weeks following treatment. Control animals from group 1 (n=10) exhibited a 6-fold increase in tumor weight ($p<0.01$) and extensive tumor spread at 4 weeks (tumor score 15.5/16), relative to pre-treatment animals sacrificed at 1 week (n=5; tumor score 2.5/16). A 10-fold increase in tumor weight ($p<0.001$ versus pre-treatment) and further tumor spread (tumor score 16/16) was noted at 7 weeks (n=10). Control animals from group 2 (n=10) exhibited a 6.5-fold increase in tumor weight ($p<0.001$ versus pre-treatment) and extensive tumor spread (tumor score 15.9/16) at 4 weeks. A 12-fold increase in tumor weight ($p<0.001$ versus pre-treatment) and diffuse tumor spread (score 16/16) were noted at 7 weeks (n=10) in these animals. There was no significant difference in tumor growth or tumor spread between the control groups receiving media or irradiated PA-1 cells.

Table 3

Tumor Weight, Milligrams

Pre-Treatment	Treatment Group Number	4 Weeks Post-Treatment	7 Weeks Post-Treatment
0.214 ± 0.032	1	1.225 ± 0.09	2.256 ± 0.06
	2	1.412 ± 0.1	2.494 ± 0.16
	3	0.225 ± 0.003	0.380 ± 0.06
	4	0.185 ± 0.04	0.131 ± 0.09

Animals treated with HSV-1716 (group 3, n=10) exhibited significantly less tumor growth ($p<0.001$) and spread (tumor score 2.5/16) at 4 or 7 weeks than control group 1. The mice of group 3 exhibited no difference in tumor weight at 7 weeks, relative to pre-treatment animals ($p=0.61$), suggesting stabilization of disease. Animals receiving HSV-infected producer cells (group 4) also had significantly smaller tumors ($p<0.001$) and less tumor spread (score 2/16) at 4 and 7 weeks than their corresponding controls (group 2). The mice of group 4 exhibited no tumor progression at 4 weeks ($p=0.43$), and significant tumor regression by week 7 ($p<0.05$ versus pre-treatment). In addition, the mice of group 4 exhibited a significantly smaller tumor burden at 7 weeks than HSV-1716-treated animals (group 3, $p<0.05$). Remarkably, in the group receiving HSV-infected PA-1 producer cells, 20% of the animals displayed no detectable EOC, either grossly or under the dissecting microscope at 4 weeks (n=4/20) or at 7 weeks (n=4/20).

In order to assess the carcinogenic potential of PA-1 producer cells, 6- to 8-week-old SCID mice (n=10) were injected intraperitoneally with 5×10^6 irradiated PA-1 cells infected with HSV-1716 at 2 MOI. The mice were observed for 16 weeks and then sacrificed in order to evaluate the presence of intraperitoneal tumors. No microscopic or gross tumor could be detected. In addition, 10 animals were injected in the flank with 5×10^6 PA-1 cells, which were prepared in a similar manner. Similarly, no tumor was detected over 16 weeks.

Use of HSV-Infected Producer Cells Prolongs the Survival of SKOV3 Tumor-Bearing Mice

Survival experiments performed using animals bearing SKOV3 tumors confirmed the oncolytic activity of HSV-1716 against EOC cells. A single dose of HSV-1716 was administered four weeks following the injection of tumor cells, a time at which there was a sizable intraperitoneal tumor burden. Administration of HSV-1716 resulted in approximate doubling of the mean survival time (17 weeks; n=20), relative to control animals, which received virus-free medium (9 weeks; n=20; $p<0.001$). Moreover, administration of HSV-infected PA-1 cells (n=20), as described

herein, slightly extended the mean survival time, relative to the survival time corresponding to administration of HSV-1716 (20 weeks; $p<0.01$ versus HSV-1716 alone).

Similar experiments were performed in xenografted mice bearing A2780 tumors. Injection of a single dose of HSV-1716 four weeks following injection of tumor cells, a time at which there was extensive intraperitoneal tumor, led to doubling of animal survival (13 weeks, n=20), relative to controls (6 weeks, n=20). Administration of PA-1 cells infected with HSV-1716 to such animals did not further extend the mean animal survival time (13.5 weeks, n=20), relative to administration of HSV-1716 alone ($p=0.987$). However, when moribund animals were sacrificed in the treated groups, the amount of intraperitoneal tumor encountered was minimal in HSV-treated animals (tumor weight 1.9 ± 0.4 grams; n=4), but even smaller in animals which were administered PA-1 cells infected with HSV-1716 (tumor weight 0.9 ± 0.4 grams, n=3). These (PA-1/HSV) animals exhibited large tumors at the abdominal injection site, which were ulcerated and necrotic, suggesting that death might have ensued due to infectious or tumor-related complications stemming from the injection site tumors. It is possible that survival would have been improved in the absence of these subcutaneous tumors. This was in sharp contrast to the untreated animals, which exhibited very bulky intraperitoneal tumors (tumor weight 22.7 ± 3.1 grams, n=4), most of which were associated with hemorrhagic ascites.

HSV-1716 Penetrates Deeply in Tumor Nodules and Causes Extensive Necrosis in a Dose-Dependent Manner

Microscopic examination of control non-treated tumors revealed solid sheets of tumor tissue having well-established vascularization. Examination of this tissue under higher magnification revealed numerous mitoses, indicating rapid tumor growth, many capillaries, and no inflammatory infiltrate. On the contrary, tumors obtained from animals treated with HSV-1716 or with HSV-infected producer cells exhibited extensive necrosis. Examination of these tumor tissues at high power revealed extensive cell vacuolization, suggesting HSV-induced CPE. Occasional

polymorphonucleate leukocytes were also visible within the necrotic areas of the tumor. Immunohistochemical detection of HSV revealed the presence of virus within the necrotic areas, wherein many vacuolized cells stained strongly for HSV. Cells located near, but not within, necrotic areas exhibited characteristic signs of cell death did not stain positive for the presence of HSV, suggesting operation of a bystander killing effect associated with HSV in these cells.

In HSV-treated animals (i.e. those treated with virus alone and those treated with virus-infected producer cells), the virus was present deep within solid tumor nodules. In distinct areas of the tumor nodules, viral spread was detectable several weeks following a single intraperitoneal injection. Immunohistochemical evaluation of tumors obtained from animals treated with HSV-infected PA-1 cells indicated that even larger areas of the tumor nodules were subject to viral infection, relative to animals treated with HSV-1716 alone. Areas of viral infection were primarily found in the interface between uninfected tumor and areas of necrosis, suggesting that, by replicating, the virus advanced into the tumor leaving behind an area of necrosis. In HSV-treated mice, the virus could not be detected in normal murine tissues such as liver, pancreas, kidney, adrenal, spleen, small bowel myenteric plexus, or brain.

The results of the experiments presented in this Example demonstrate that intraperitoneal administration of HSV-1716 to tumor-bearing animals results in significant reduction or arrest of EOC tumor growth. The effect of a single intraperitoneal dose to stabilize intraperitoneal disease was noted even with bulky tumors and with different cell lincs. Those results merely confirm what was hypothesized in the prior art. However, the results of the experiments presented in this Example also demonstrate that administration of producer cells infected with an oncolytic virus such as HSV-1716 is at least as, if not more, effective for killing tumor cells in an animal and preventing tumor growth and spread. These experiments therefore represent a significant advance over prior art methods of delivering oncolytic viruses to subjects.

These results also demonstrate that delivery of oncolytic virus using producer cells resulted in infection of larger areas of intraperitoneal tumors by the virus and in deeper penetration of the virus within tumor nodules, relative to virus administered by direct injection. Without wishing to be bound by any particular theory of operation, it is understood that two possible mechanisms may account for the improved virus delivery effected by use of producer cells. First, based on the *in vitro* observations described herein, lysis of infected producer cells within the peritoneal cavity may have released large amounts of viral particles within the peritoneal fluid. Second, since HSV is known to propagate by direct cell-to-cell spread (Dingwell et al., 1994, J. Virol. 68:834-845; Weeks et al., 1997, Biochem. Biophys. Res. Commun. 235:31-35), producer cells may have promoted direct cell-to-cell viral infection of tumor cells by binding to EOC cells. PA-1 cells appeared to adhere predominantly to areas of peritoneum that were covered by established tumor and not to close-by areas of normal peritoneum. There was a close correlation between the presence of tumor and PA-1 cells, as calculated by odds ratios. It is possible that the physical forces governing fluid migration within the peritoneal cavity were partially responsible for this effect or that species-related differences in the affinity of interaction might have influenced these findings. However, the *in vitro* studies performed using human normal mesothelial cells demonstrated that PA-1 cells bound predominantly to EOC surfaces compared to normal human mesothelium. This suggests that cell-cell interactions promote adhesion of PA-1 producer cells to tumor cells, and that such adhesion promotes transfer of virus from producer cells to tumor cells.

It may be that, by virtue of viral amplification and through direct adhesion of producer cells to tumor cells, a significantly larger amount of virus infected the intraperitoneal tumor cells. Although immune neutralization of HSV was not an issue with the SCID model, this may occur in the peritoneal cavity of an immunocompetent host by complement or neutralizing antibodies, which may be present in the peritoneal fluid. Indeed, anti-herpes antibodies are highly prevalent in the adult population. Use of producer cells to deliver oncolytic virus might partly

circumvent this immune barrier because cell-to-cell spread is less affected by neutralizing antibodies than infection by free virus.

Example 3

Mutant Type-1 Herpes Simplex Virus Therapy Prolongs Survival in a Murine Intraperitoneal Tumor Model

HSV-1716, a replicating herpes simplex type 1 virus with a mutation in the gamma 34.5 gene, is non-neurovirulent and has shown efficacy as an oncolytic treatment of mesothelioma in immunodeficient mice. To further evaluate its possible role in cancer gene therapy, an immunocompetent animal model was investigated. EJ-62, a *ras*-mutated murine fibroblast, grows well in the peritoneum of immunocompetent BALB/c mice and, unlike most murine cells, is sensitive to herpesvirus infection. The effects of HSV-1716 in this syngeneic intraperitoneal model, which resembles mesothelioma and ovarian cancer, were studied, and the efficacy of both single and multiple virus injections of virus were evaluated. Use of producer cells in this model was also investigated.

Producer cells were infected *ex vivo* with the virus, irradiated, and then injected intraperitoneally. Cell viability studies were performed on EJ-62 at varying MOI values. At an MOI of 1 or greater, greater than 90% cell death, relative to control cells, was observed. MOI values as low as 0.01 resulted in approximately 75% cell death, relative to control cells.

Established intraperitoneal tumors were treated with virus injections in a single (4×10^6 particles) dose, multiple doses (three doses given every fourth day), or a single comparable dose of producer cells. Prolonged survival was observed in all treated groups, relative to control cells. The median survival of control cells was about 30 days. The median survival of cells treated with a single viral injection was about 62 days. Cells treated with multiple virus injections exhibited >60% survival at 70 days. Cells treated with a single dose of producer cells exhibited >65% survival at 70 days.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

What is claimed is:

1. A producer cell for administration to a subject having tumor cells, said producer cell comprising an oncolytic virus, wherein said oncolytic virus is capable of replicating in said producer cell, said producer cell being incapable of sustained survival in the body of the subject.

2. The producer cell of claim 1, wherein said oncolytic virus is cytotoxic with respect to said producer cell in the body of the subject.

3. The producer cell of claim 1, wherein said producer cell is rendered incapable of sustained survival in the body of the subject by exposing said producer cell to a lethal dose of radiation.

4. The producer cell of claim 3, wherein said lethal dose of radiation is a dose which enhances the burst size of said producer cell.

5. The producer cell of claim 4, wherein said dose of radiation is about 3 Gray.

6. The producer cell of claim 1, wherein said producer cell is rendered incapable of sustained survival in the body of the subject by incorporating a suicide gene into said producer cell.

7. The producer cell of claim 6, wherein said suicide gene encodes a protein selected from the group consisting of thymidine kinase and cytosine deaminase.

8. The producer cell of claim 1, wherein said producer cell exhibits binding affinity for a tumor cell in the subject.

9. The producer cell of claim 8, wherein the tumor cell is an epithelial tumor cell.

10. The producer cell of claim 9, wherein the epithelial tumor cell is an epithelial ovarian cancer cell.

11. The producer cell of claim 1, wherein said oncolytic virus is capable of replicating in a tumor cell of the subject.

12. The producer cell of claim 11, wherein said oncolytic virus is less capable of replicating in a non-tumor cell of the subject than in the tumor cell.

13. The producer cell of claim 1, wherein said oncolytic virus is incapable of replicating in a non-tumor cell of the subject.

14. The producer cell of claim 13, wherein replication of said oncolytic virus is under the control of a tumor-associated transcriptional promoter.

15. The producer cell of claim 14, wherein said transcriptional promoter is selected from the group consisting of the prostate specific antigen promoter and the tumor growth factor-β promoter.

16. The producer cell of claim 1, wherein said oncolytic virus is incapable of replicating in any cell of the subject.

17. The producer cell of claim 1, wherein said producer cell is selected from the group consisting of a PA-1 cell, an REN cell, a PER.C6 cell a 293 cell, a melanoma cell, a glioma cell, and a teratocarcinoma cell.

18. The producer cell of claim 17, wherein said producer cell is a PA-1 cell.

19. The producer cell of claim 1, wherein said oncolytic virus is selected from the group consisting of a herpes simplex virus-1, a herpes simplex virus-2, an adenovirus, a vesicular stomatitis virus, a Newcastle disease virus, and a vaccinia virus.

20. The producer cell of claim 19, wherein said herpes simplex virus-1 does not express functional ICP34.5.

21. The producer cell of claim 19, wherein said herpes simplex virus-1 is selected from the group consisting of HSV-1716, HSV-3410, HSV-3616, HSV-R3616, HSV-R47, HSV-G207, HSV-7020, HSV-NVR10,, HSV-G92A, HSV-3616-IL-4, and HSV-hrR3.

22. The producer cell of claim 19, wherein said herpes simplex virus-2 is selected from the group consisting of strain 2701, strain 2616, and strain 2604.

23. The producer cell of claim 19, wherein said adenovirus is selected from the group consisting of ONYX-15, Ad5d1520, Ad5d1312, CN706, Addl110, Addl111, Addl118, and Addl004.

24. The producer cell of claim 1, further comprising a composition selected from the group consisting of an immunomodulatory molecule, a cytokine, a targeting molecule, a cell growth receptor, an immunoglobulin which is specific for the tumor, a nucleic acid encoding an immunomodulatory molecule, a nucleic acid encoding a cytokine, a nucleic acid encoding a targeting molecule, a nucleic acid encoding a cell growth receptor, and a nucleic acid encoding an immunoglobulin which is specific for the tumor.

25. An anti-tumor agent comprising a mammalian cell which comprises thymidine kinase, wherein said mammalian cell exhibits binding affinity for a tumor cell in a human patient and is incapable of sustained survival in the body of the patient, whereby when said mammalian cell is administered to the patient, said mammalian cell binds with a tumor cell in the patient, and when gancyclovir is thereafter administered to the patient, said mammalian cell metabolizes gancyclovir to generate a cytotoxic metabolite which is provided to the tumor cell with which the mammalian cell has bound.

26. A method of killing tumor cells in a mammal, the method comprising administering to the mammal a producer cell, said producer cell comprising a oncolytic virus, wherein said oncolytic virus is capable of replicating in said producer cell, said producer cell being incapable of sustained survival in the body of the mammal.

27. The method of claim 26, wherein said mammal is a human afflicted with an epithelial cancer.

28. The method of claim 27, wherein said mammal is a human afflicted with an tumor.

29. Use of a producer cell for manufacture of a medicament for administration to a patient having tumor cells, said producer cell comprising a oncolytic virus, wherein said oncolytic virus is capable of replicating in said producer cell, said producer cell being incapable of sustained survival in the body of the patient.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05466

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : A01N 63/00; C12N 1/20, 15/00; C12P 21/06
US CL : 435/ 252.3, 320.1, 69.1; 424/93.21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/ 252.3, 320.1, 69.1; 424/93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS
replication competent herpes simplex type 1 virus transform transfect cancer tumour tumor melanoma pa-1 cell hsv

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,585,096 A (MARTUZA et al.) 17 December 1996, see entire reference.	1, 2, 7-18, 19-29
Y	US 5,601,818 A (FREEMAN et al.) 11 February 1997, see entire reference.	1-29

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
03 JUNE 1999

Date of mailing of the international search report
07 JUL 1999

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05466

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/05466

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-24, 26-29, drawn to a producer cell, a method of using the producer cell and a method of making the producer cell.
Group II, claim(s) 25, drawn to an anti-tumor agent which is a mamalian cell which comprises thymidine kinase.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

A national stage application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept. If multiple products, processes of manufacture or uses are claimed, unity of invention is determined by 37 CFR 1.475(b) which defines the combinations of categories held to have unity of invention. See PCT article 17(30(a) and 1.476 (c), 37 CFR 1.475 (b). After that all other products and processes will be broken out as separate inventions.

Group I is drawn to a producer cell, a method of making and a method of using said cell.

Group II is drawn to an additional product.